JOURNAL OF VIROLOGY, June 1988, p. 1917–1924
0022-538X/88/061917-08$02.00/0

Vol. 62, No. 6

Characterization of Primary Human Keratinocytes Transformed by Human Papillomavirus Type 18

PRITINDER KAUR* AND JAMES K. McDOUGALL
Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104

Received 9 November 1987/Accepted 22 February 1988

Primary human epithelial cells were cotransfected with pHPV-18 and pSV2neo, and cell strains were generated by selecting in G418. One cell strain (FE-A), which exhibits an extended life span, is currently in its 30th passage. In comparison, control cultures can only be maintained up to the seventh passage. Southern blot analysis revealed the presence of at least one intact, integrated viral genome in these cells. FE-A cells showed altered growth properties, characterized by a change in morphology, and clonal density. Differentiation markers analyzed by Western blotting (immunoblotting), such as cytokeratins and involucrin, indicated that the cells resembled a partially differentiated epithelial population. Increased expression of the 40-kilodalton cytokeratin was observed in FE-A cells, similar to that observed in simian virus 40-immortalized human keratinocytes (M. Steinberg and V. Defendi, J. Cell Physiol. 123:117–125, 1985). FE-A cells were also found to be defective in their response to terminal differentiation stimuli. Calcium and 12-*O*-tetradecanoyl-phorbol-13-acetate treatment induced normal epithelial cells to differentiate, whereas the human papillomavirus 18 (HPV-18)-containing keratinocytes were resistant to these signals, indicating their partially transformed nature. These cells were not able to induce tumors in nude mice over a period of up to 8 months. A second cell strain, FE-H18L, also generated by transfecting HPV-18, also exhibited an extended life span and similar alterations in morphology. Viral RNA transcribed from the early region of HPV-18 was detected in both cell strains by Northern (RNA) blot analysis. These cell strains should provide a useful model for determining the role of HPV in carcinogenesis.

Papillomaviruses are species-specific viruses infecting a wide range of vertebrates, including amphibians, reptiles, birds, and a variety of mammals (20, 30). These viruses are etiologic agents of benign cutaneous and mucosal epithelial lesions termed papillomas or fibropapillomas (reviewed in references 24, 30, and 54). To date, over 50 distinct types of human papillomaviruses (HPVs) have been identified, a new type being defined as one that reveals less than 50% homology with known virus types by nucleic acid hybridization. It is now evident that HPVs are closely associated with malignant disease. Molecular cloning of various HPVs has allowed the detection of viral DNA in a variety of benign and malignant epithelial lesions, and the presence of HPV DNA has been particularly well documented for the female genital tract epithelium. Individual HPVs show a preference for epithelia from specific anatomical sites, and thus only a few of the characterized types are associated with genital lesions. These include HPV-6, -11, -16, -18, -31, -33, -34, and -35. Of these, HPV-6 and HPV-11 are most often observed in benign genital warts in both males and females and in mild or moderate cervical dysplasias (12–14). HPV-16 and HPV-18 are the most frequently detected genomes in cervical and penile cancer biopsies (3, 9, 14), with 60 to 90% of malignant cervical carcinomas containing HPV-16 and 15 to 36% containing HPV-18 (reviewed in references 24 and 31). In addition, a number of cell lines derived from cervical carcinomas contain either HPV-16 (SiHa and CaSki) or HPV-18 (HeLa, SW756, C4-I, and C4-II) DNA with a subset of viral genes transcribed (35, 37, 48), suggesting that viral products may have a role in transformation.

While there is a great deal of circumstantial evidence for the role of HPVs in carcinogenesis, an experimental basis for HPV oncogenicity in a suitable in vitro model system remains to be established. This is primarily due to the lack of an in vitro culture system for the propagation of these viruses. However, the availability of recombinant viral DNA has allowed the initiation of transformation studies with HPVs. Morphologic transformation of C127 cells with HPV-1 and HPV-5 has been demonstrated (42). More recently it has been shown that HPV-16, when cloned as a head-to-tail dimer into the pSV2neo plasmid, induced transformation of NIH 3T3 cells (47). The viral DNA was integrated and expressed in all tumorigenic lines. Similar studies have been carried out in NIH 3T3 cells with cloned HPV-6b and HPV-18 DNA in this laboratory (18).

Given the epitheliotropic nature and anatomical site preferences of the HPVs, primary human epithelial cells from an appropriate site should provide the optimum model culture system for investigating the role of HPV in carcinogenesis. There are currently two studies reported in the literature describing the introduction of HPV-16 into primary human epithelial cells (8, 32). Both studies have demonstrated that the introduction of HPV-16 into these cells results in their immortalization. This is correlated with the integration of viral DNA into the host genome and its transcription. This paper describes the results of introducing a related HPV type, HPV-18, into primary human keratinocytes derived from neonatal foreskins.

MATERIALS AND METHODS

Isolation and transfection of human keratinocytes. In initial experiments, primary human keratinocytes isolated from neonatal foreskins with dispase (Boehringer Mannheim Biochemicals) and trypsin-EDTA (GIBCO) were plated in the basal nutrient medium MCDB 153 (Irvine Scientific) described by Boyce and Ham (5). This was supplemented with epidermal growth factor (EGF; 10 ng/ml; Collaborative Research Inc.), insulin (5 μg/ml), hydrocortisone (0.5

* Corresponding author.

μg/ml), transferrin (5 μg/ml), 0.1 mM ethanolamine and phosphorylethanolamine (Sigma Chemical Co.), and whole bovine pituitary extract (University of Colorado) (70 μg/ml). More recently, cells have been initiated and maintained in keratinocyte growth medium (KGM; Clonetics Corporation), which is modified MCDB 153 augmented with growth factors and bovine pituitary extract.

Primary human keratinocytes were harvested and subsequently plated at 5×10^5 to 6×10^5 cells per 60-mm dish or 1.5×10^6 to 2×10^6 cells per 100-mm dish, 24 h prior to transfection. Cells were briefly trypsinized 2 to 3 h before transfection. Calcium phosphate precipitates of DNAs were made as described by Wigler et al. (44) and contained 1 μg of pSV2neo and 10 μg of either salmon sperm, HPV-6b, or HPV-18 DNA. In some cases, HPV inserts were released from the plasmid by digestion with the appropriate restriction enzymes and ligated to produce concatamers or circles. The levels of calcium and phosphate in MCDB 153 make this medium unsuitable for calcium phosphate DNA precipitation; therefore, 0.5 ml of the precipitate was added to the cells for 15 to 20 min at room temperature and then Dulbecco modified Eagle medium (GIBCO) containing 10% chelated fetal calf serum was added to the cells for 3 to 4 h at 37°C. Cells were shocked with 15% glycerol in transfection buffer (HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-buffered saline [HBS], pH 7.1) for 2 min, washed with HBS, and refed fresh KGM. Selection in KGM containing 50 to 100 μg of G418 per ml was carried out at 48 h posttransfection for a week or longer.

Analysis of viral DNA and RNA. High-molecular-weight DNA was isolated from cells, digested with various restriction enzymes, electrophoresed, and blotted by standard procedures (22). Hybridization with the ^{32}P-labeled nick-translated HPV-18 insert released from the vector was carried out at T_m −30°C, and the filters were washed stringently at T_m −22°C.

RNA was extracted in guanidium isothiocyanate and centrifuged into a cesium cushion by standard techniques (22). RNA was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose. Hybridization with the ^{32}P-labeled nick-translated *Eco*RI-*Bam*HI 2.35-kilobase (kb) fragment of HPV-18 encoding the E6, E7, and two-thirds of the E1 region was carried out at T_m −18°C, and filters were washed at T_m −17°C.

Western blot (immunoblot) analysis of cytokeratins and involucrin. Keratinocytes were scraped into urea extraction buffer (8 M urea, 50 mM Tris [pH 7.6], 10 mM dithiothreitol, 7 μl of 2-mercaptoethanol per ml, 20 trypsin-inhibitor units of aprotinin per ml) and sonicated. Total protein content of the cell extracts was quantitated by the Bradford assay (6) with bovine serum albumin (Bethesda Research Laboratories, Inc.) as a standard for calibration. An equal volume of Laemmli protein sample buffer was added to 10 μg of protein extract per sample, and the mixture was heated at 100°C for 5 min. Prestained proteins (Bethesda Research Laboratories) were used as molecular weight markers. Proteins were transferred to nitrocellulose electrophoretically in 25 mM Tris–195 mM glycine (pH 8.5)–20% methanol at 50 V overnight. The nitrocellulose filter was soaked in 10 mM *N*-ethylmaleimide in phosphate-buffered saline (PBS) for 10 min and then blocked with Blotto (5% nonfat dry milk, 0.9% NaCl, 0.1% Antifoam-A [Sigma] and 0.1% sodium azide) for 30 min. Incubations with primary antisera were carried out in Blotto at 4°C overnight. For cytokeratin localization, the monoclonal antibodies AE1 and AE3 (Hybritech Inc.) were used at a dilution of 1:1,000 as primary antibodies and goat anti-mouse immunoglobulin G (IgG) conjugated to ^{125}I (New England Nuclear) at a dilution of 1:1,000 as a secondary antibody for 2 to 3 h at room temperature in Blotto. The filters were washed after incubation with primary and secondary antisera three times for 20 min each with 0.5% deoxycholate–0.1 M NaCl–0.5% Triton X-100–10 mM sodium phosphate, pH 7.4.

The localization of involucrin could be carried out on the same filter by immunohistochemical techniques. Briefly, the blot was incubated in rabbit anti-human involucrin antisera (Biomedical Technologies, Inc.) for 60 min, washed in 0.05% Tween in PBS, incubated in goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega Biotech) at a dilution of 1:7,500 for 2 h at room temperature, rinsed in alkaline phosphatase buffer (0.1 M Tris [pH 9], 0.1 M NaCl, 5 mM $MgCl_2$), and then stained with 8 mg of nitroblue tetrazolium and 8 mg of 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidene salt (Sigma) in 50 ml of alkaline phosphatase buffer for 5 min until color development was complete.

Ca^{2+} and TPA studies. Cells were plated in KGM at 2×10^5/100-mm plate and allowed to reach exponential log phase. The medium was replaced with KGM containing a final concentration of 100 nM 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA; Sigma) in acetone, and the cells were observed for up to 10 days. Protein extracts were made at various sampling times. The concentration of calcium in the KGM was raised by adding calcium chloride to a final molarity of 1.2 mM to the medium. Morphological changes and cytokeratin expression were studied.

Tumorigenicity studies. Cells were trypsinized and resuspended in PBS. Four BALB/c *nu/nu* male mice between 3 and 4 weeks old were injected with 5×10^6 cells per 0.1 ml of PBS subcutaneously per cell strain tested. All mice were injected on the right flank and monitored twice weekly for the appearance of tumors over a period of 3 to 4 months.

RESULTS

Growth properties and phenotype of HPV-18-transfected keratinocytes. In one experiment the cotransfection of human neonatal foreskin keratinocytes with pHPV-18 (see Fig. 2a) and pSV2neo and subsequent selection in medium containing G418 resulted in the generation of one cell strain, FE-A, originating from a G418-resistant colony. A second experiment, in which primary keratinocytes were cotransfected with pSV2neo and HPV-18 DNA consisting of the insert from pHPV-18 ligated to itself (to complete the interruption in the E1 open reading frame resulting from cloning into the *Eco*RI site of pBR322), generated 29 resistant colonies with 30 μg of HPV-18 and 3 μg of pSV2neo. One cell strain, designated FEH18L, was derived from this experiment by pooling approximately 10 G418-resistant colonies. In both experiments, transfections were also carried out with HPV-6b and pSV2neo, pSV2neo only, or salmon sperm DNA only. Although G418-resistant colonies were obtained with plasmid or released and religated HPV-6b or pSV2neo transfections, these were not able to grow sufficiently for maintenance and analysis. This suggests that the introduction of HPV-18 DNA into primary human keratinocytes confers a growth advantage on these cells. Indeed, both the FE-A and FEH18L strains exhibited an extended life span in culture. The FE-A cell strain has been in culture for 14 months and FEH18L for 11 months. Control cultures can be maintained in vitro for approximately 3 months.

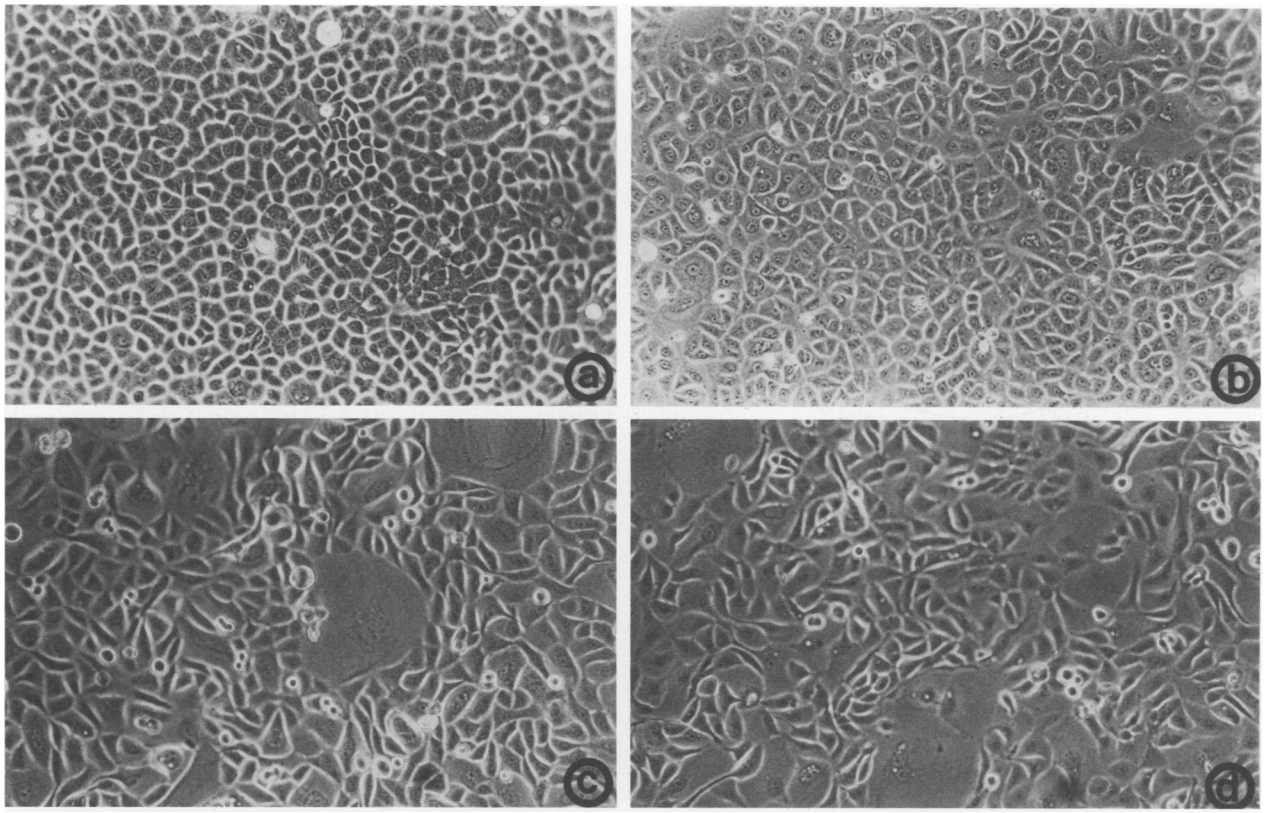

FIG. 1. Morphology of normal (a), pSV2neo-transfected (b), and HPV-18-transfected (c and d) human keratinocytes. The FE-A cell strain (c) and FEH18L cell strain (d) transfected with HPV-18 show distinct alterations in morphology and grew less densely. Phase contrast micrographs; ×92.

Salmon sperm-transfected keratinocytes (not selected in G418) can also be maintained for about 3 months but undergo fewer population doublings. Growth curves of FE-A and control keratinocytes indicate that the length of the cycle is similar in both HPV-18-containing and normal epithelial cells, i.e., approximately 35 h.

FE-A cells were tested for the presence of cytokeratins at passage 7 and FEH18L cells were tested at passage 17 to ensure that they were of epithelial origin by staining with a polyclonal antiserum to bovine hoof keratin (Miles-Yeda Ltd.). All cells were positive, exhibiting fibrillar staining in the perinuclear region and throughout the cytoplasm (data not shown).

Both FE-A and FEH18L cells exhibited altered morphology in culture compared with pSV2neo-transfected cells or untransfected cells (Fig. 1). In general, HPV-18-transfected cells showed a lower cell density at confluence than the control and lost their polygonal shape, growing in a more disorganized manner.

Viral DNA and RNA. High-molecular-weight DNA was extracted from FE-A cells at passage 5 and analyzed by Southern blotting (Fig. 2b). The high-molecular-weight band in the undigested DNA suggested that all the viral DNA was integrated into chromosomal DNA. The *Eco*RI digest yielded a single band at 7.9 kb, indicating that intact copies of viral DNA were retained in this cell strain and that integration occurred within the pBR322 moiety of the plasmid. *Bgl*I digestion of FE-A DNA suggested that the *Bgl*I sites at nucleotides 928 or 1162 and 3481 in pBR322 were retained as well as the *Bgl*I site at nucleotide 7576 of HPV-18 (Fig. 2a), so that a *Bgl*I digest yielded two fragments of 6 and approximately 4 kb (Fig. 2b). The additional bands in the *Bgl*I-digested DNA may indicate a second insertion of HPV DNA, but more likely represent partial digestion products. The FEH18L cell strain also contained HPV-18 DNA detected by Southern blot, retaining an *Eco*RI site within the integration (data not shown).

Total RNA isolated from FE-A, FEH18L, and control cells was probed for the presence of viral transcripts with the nick-translated 2.35-kb HPV-18 DNA fragment containing the E6, E7, and most of the E1 region. Figure 3 shows the autoradiogram of the Northern blot. Clearly, a number of viral transcripts were present in both HPV-18-transfected cell strains (FE-A and FEH18L), ranging from less than 2.0 to about 6.0 kb in size. Presumably, some of the larger species of RNA are chimeric virus-cell fusion transcripts, similar to those reported by Schwarz and co-workers for cervical carcinoma cell lines (35). The viral transcripts present in the two cell strains FE-A and FEH18L were of different sizes, which may indicate inherent differences in transcription between these two cell strains.

Western blot analysis of cytokeratins. Figure 4 shows the autoradiograms obtained from Western blots of normal, differentiating, and FE-A cells. Primary human keratinocytes grown in MCDB-153 or KGM contained five distinct cytokeratins recognized by the AE1 and AE3 monoclonal antibodies: these were 40 kDa (K19, Moll classification [25]), 48 kDa (K16), 50 kDa (K14), 56 kDa (K6), and 58 kDa (K5). When keratinocytes were maintained in culture past confluence in KGM, stratification of cells was observed as the cells began to differentiate. Concurrently, quantitative changes in the cytokeratins were observed (Fig. 4). The induction of

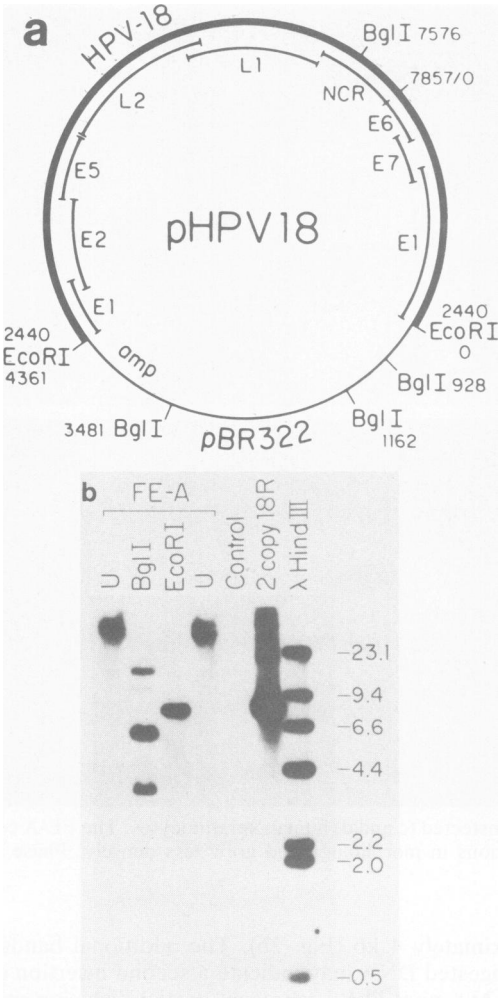

FIG. 2. (a) Map of pHPV-18 showing *Bgl*I and *Eco*RI sites within the plasmid (numbers indicate corresponding nucleotide sites). The inner lines indicate the positions of the open reading frames within the 7,857-bp HPV-18 genome. NCR, Noncoding region. Note the interruption of the E1 open reading frame due to cloning at the *Eco*RI site of pBR322. (b) Southern blot analysis of HPV-18 DNA content of FE-A cells. U, Undigested high-molecular-weight DNA; BglI and EcoRI, restriction enzyme digests; control, untransfected epithelial cell DNA; 2 copy 18R, reconstruction of viral DNA; λ HindIII, end-labeled molecular weight marker DNA. The probe consisted of the nick-translated insert of pHPV-18. Sizes are shown in kilobases.

differentiation in subconfluent cultures by the addition of 100 nM TPA or calcium to a final molarity of 1.2 mM also resulted in alterations in the relative amounts of cytokeratins present in these cells (Fig. 4). In particular, the higher-molecular-weight species were less abundant in cells induced to differentiate by TPA or high calcium. The cytokeratins expressed by the FE-A cells are also shown in Fig. 4 and were clearly different from those of control, mitotically active keratinocytes. The 40-kDa species was clearly overexpressed, while the higher-molecular-weight cytokeratins were downregulated. Indeed, these HPV-18-containing cells had a cytokeratin profile similar to that of partially differentiated keratinocytes, in particular one comparable to that induced by TPA treatment of normal keratinocytes. Changes in cytokeratin expression were not seen in salmon sperm-transfected controls (Fig. 4).

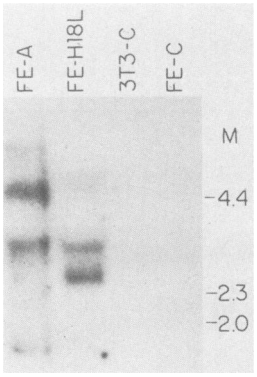

FIG. 3. Northern blot analysis of HPV-18-transfected (FE-A and FEH18L) and control cells. Total RNA was loaded at 10 μg per lane. The probe was the 2.35-kb *Eco*RI-*Bam*HI fragment of pHPV-18 encoding the E6, E7, and 5′ two-thirds of the E1 gene. Untransfected controls: 3T3-C, NIH 3T3 cells; FE-C, epithelial cells; M, λ HindIII marker DNA. Sizes are shown in kilobases.

Western blot analysis of involucrin. Keratinocytes form an insoluble protein envelope beneath the plasma membrane as part of the terminal differentiation process Involucrin is a soluble protein precursor of the cross-linked envelope (34) and provides an additional differentiation marker for epithelial cultures. Immunohistochemical localization of involucrin with secondary antibodies conjugated to alkaline phosphatase was carried out on the same nitrocellulose filters probed for cytokeratins. Control cultures of keratinocytes showed a characteristic doublet of bands at just over 97 kDa (Fig. 5). Involucrin has been estimated to be 92 kDa (34), but the mobility of this protein varies with the electrophoretic separation conditions. The positions of the prestained molecular

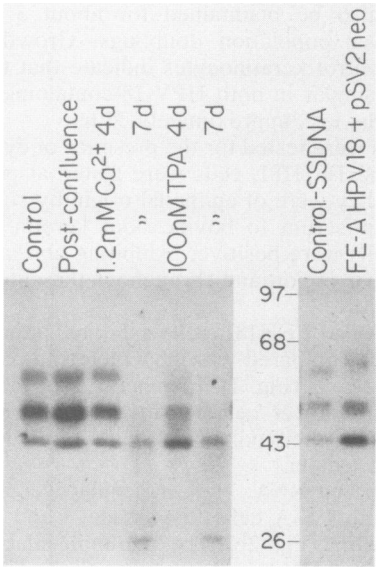

FIG. 4. Western blot analysis of cytokeratins. (Left) Keratins present in control (subconfluent), postconfluent differentiating, and calcium- or TPA-stimulated (4 or 7 days) differentiating human keratinocytes. (Right) Cytokeratins present in HPV-18-containing FE-A cells, together with a transfection control cell strain which received salmon sperm (SS) DNA. The AE1 and AE3 antibodies used in this blot allowed the detection of 40-, 48-, 50-, 56-, and 58-kDa cytokeratins. The positions of prestained markers are indicated (in kilodaltons) between the panels.

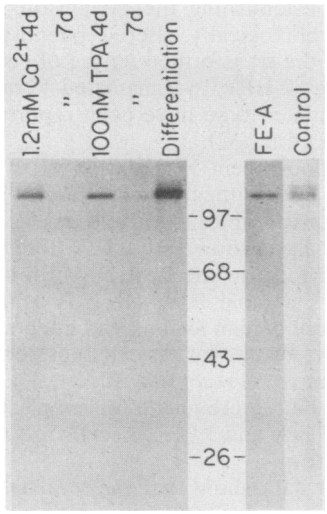

FIG. 5. Western blot analysis of involucrin in control (subconfluent), postconfluent differentiating, calcium- or TPA-induced differentiating, and HPV-18-containing FE-A keratinocytes. See Fig. 4 legend for details.

weight markers shown in Fig. 4 and 5 cannot be used in an absolutely quantitative manner, since coupling with the blue chromophore dye affects their electrophoretic mobility. The maintenance of keratinocytes beyond confluence and hence stratification resulted in accumulation of involucrin (Fig. 5). A single band was observed in this case. Stimulation of control cultures with 100 nM TPA or increased calcium affected the involucrin content of epithelial cells (Fig. 5). Typically, the characteristic doublet band seen in subconfluent cultures was replaced by a single band. FE-A cells showed an involucrin content similar to that of calcium- or TPA-stimulated differentiated control cultures (Fig. 5).

Response of FE-A cells to differentiation stimuli. Primary and early-passage keratinocytes were tested for their response to increased calcium concentration in the growth medium by adding calcium chloride to give a final molarity of 1.2 mM. Changes in morphology of the keratinocytes were observed within 24 h, followed by ordered stratification and differentiation in subsequent days. Figure 6a shows these changes in normal cells. FE-A cells also showed an alteration in morphology somewhat characteristic of increased calcium levels when grown in 1.2 mM calcium-containing medium (Fig. 6b). However, terminal differentiation was not observed in these HPV-18-containing cells. Normal human epithelial keratinocytes appeared to be irreversibly commit-

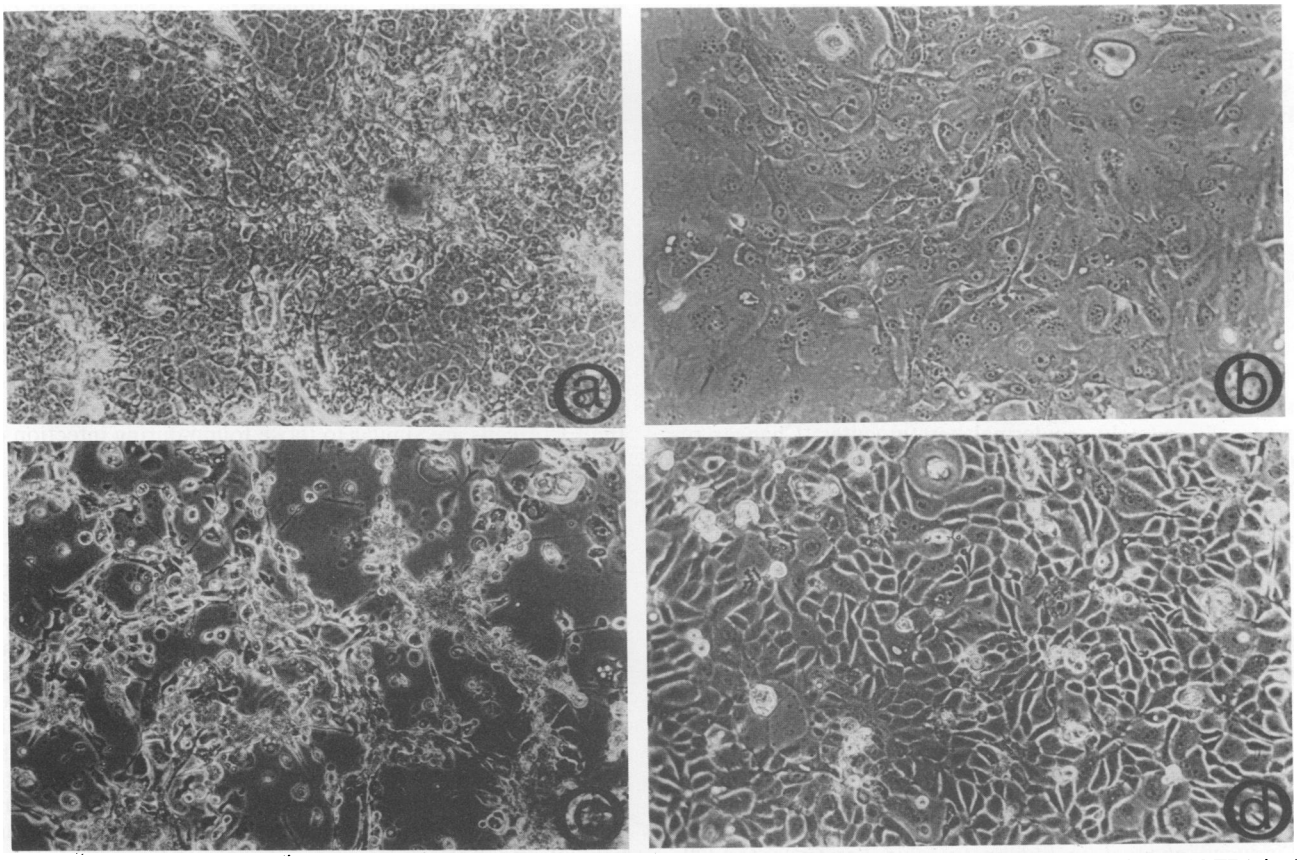

FIG. 6. Response of normal and FE-A cells to the calcium stimulus for differentiation (a and b) and to the addition of 100 nM TPA in the growth medium (c and d). Normal cells (a) undergo irreversible terminal differentiation, resulting in stratification and sloughing off of cells. HPV-18-transfected FE-A cells (b) show an alteration in morphology in response to increased calcium (1.2 mM) in the growth medium. Cells become flattened, spread out, and remain quiescent. Returning FE-A cells to normal, lower calcium levels (0.1 mM) results in the resumption of proliferative activity. Normal cells (c) undergo terminal differentiation within 7 to 10 days of TPA addition and are lost from culture. FE-A cells (d) show a heterogeneous response to TPA. A proportion of FE-A cells differentiate (not shown here); however, many cells are able to resist the stimulus to differentiate and can be maintained in culture in the presence of TPA. Phase constrast micrographs; × 90.

ted to differentiation in the high-calcium medium and continued to be lost to terminal differentiation even after they had been returned to regular (low-calcium) KGM. In contrast, FE-A cells were able to resume their former morphology and proliferative activity and could be maintained indefinitely thereafter.

Normal untransfected and salmon sperm DNA-transfected keratinocytes were treated with 100 nM TPA, and their differentiation response was investigated. Morphological changes were observed within 24 h, and cells became flattened and dendritic in appearance. Subsequently, suprabasal migration of keratinocytes was apparent, and the cultures acquired lattice works of differentiating cells (Fig. 6c). Treatment of FE-A cells with identical doses of TPA yielded a heterogeneous response. Some of the HPV-18-containing cells responded by terminally differentiating, comparable to controls, while a significant number were able to resist TPA signals and maintain themselves in culture (Fig. 6d). Indeed, a small proportion of cells appeared to be stimulated to proliferate in the presence of TPA. FE-A cells surviving the TPA stimulus to differentiate after 10 days of treatment with 100 nM TPA were pooled and maintained as a separate cell strain (FE-AP^r), and their tumorigenicity was tested in nude mice. Initial experiments showed that the FEH18L cell strain was also TPA resistant and that maintenance of prolonged 100 nM TPA-treated FEH18L cells is possible. Control cultures were routinely lost to terminal differentiation after the same period of treatment with TPA.

Tumorigenicity in nude mice. The FE-A, FE-AP^r, and FEH18L cell strains and untransfected keratinocytes were tested for tumorigenicity in nude mice. Mice injected with FE-A cells at passage 8 have been monitored for 8 months to date without the appearance of tumors. FE-AP^r cells were expanded by passaging twice and injected into mice. No tumors have been observed to date. FEH18L cells have also not been tumorigenic after a period of 4 months. Control keratinocytes did not generate tumors after being monitored for 8 months to date.

DISCUSSION

The data presented here report initial experiments in studying the role of HPVs in carcinogenesis in their in vivo target cells, namely, epidermal keratinocytes. Changes resulting from the introduction of HPV-18 into primary human epithelial cells are reported. The foremost change observed in these cells was their extended life span—the FE-A cell strain derived clonally from a single G418-resistant colony has been maintained in culture for over a year without loss through differentiation, compared with primary cultures, which can only be maintained for up to 3 or 4 months. The FEH18L strain has continued to grow, while the parent culture transfected with carrier DNA has been lost from culture 3 to 4 months after initiation. These data confirm the recent reports that HPV-16 similarly extends the life span of primary neonatal foreskin keratinocytes in vitro (8, 32). EGF-independent growth has been observed for both the FE-A and FEH18L cell strains (data not shown). These altered growth properties are analogous to simian virus 40-induced changes in human foreskin keratinocytes (39, 40). The alterations in the morphology of HPV-18-transfected cell strains reported here may reflect alterations in the cytoskeleton of these cells, resulting in loss of the polygonal packing characteristic of normal epithelial cells. Fey and Penman (11) have shown that malignantly transformed MDCK cells lose their polygonal packing and exhibit altered cytoskeletal organization, suggesting that these changes may be essential in establishing the neoplastic state. Alternatively, the morphologically altered phenotype may be a consequence of the transformed nature of cells. Changes in the morphology of HPV-16-transfected keratinocytes to a more undifferentiated type have been reported by Durst et al. (8) and Pirisi et al. (32).

The immunofluorescent localization of cytokeratins ensured that these cells with altered morphologies were indeed epithelial in nature despite extensive passage in vitro, and Southern blot analysis showed that HPV-18 DNA is retained in an integrated form in the host DNA in both FE-A and FEH18L cells. Episomal HPV DNA is usually associated with biopsies from benign lesions and integrated viral DNA with malignant lesions as well as cell lines derived from such lesions, for example, HeLa (3, 4, 10, 24, 36). Integration of viral DNA may be a prerequisite for neoplastic transformation, although FE-A and FEH18L cells are not fully transformed.

Northern blot data show that the viral DNA is actively transcribed, in particular the early region encoding the E6, E7, and E1 genes, in the cell strains described here. This part of the HPV-18 genome has been shown to be retained and transcribed in cervical carcinoma cell lines such as HeLa, C4-1, and SW756 (35) and is thought to have a role in transformation. Durst et al. (8) have also demonstrated the presence of early-region gene transcripts in their epithelial lines. A more detailed analysis of the mRNAs in our cell strains will reveal the similarities between these and carcinoma cell lines. Our data suggest that transcription of the early region of HPV-18 may not be sufficient for malignant transformation of primary epithelial cells but may be responsible for the immortalization of these cells.

The presence of karyotypic abnormalities in FE-A cells, such as aneuploidy, dicentrics, double minutes, and translocations (J. K. McDougall and P. Kaur, manuscript in preparation), are also indicative of the transformed nature of these cells. Changes in ploidy and chromosomal translocations have also been reported by Durst et al. (8) for their HPV-16-transfected cells. Anomalies of chromosome constitution are characteristic features of cervical carcinoma, and there is evidence of nonrandom involvement of chromosomes (1) in these tumors. The types of aberration seen in the FE-A cells are similar to those found during infection or after transformation by many DNA viruses (15).

The cytokeratin expression in the FE-A cell strain is clearly altered compared with control cultures. Although all the cytokeratins present in control cultures occur in FE-A cells, there are quantitative changes, such as an upregulation of the 40-kDa (K19) species and a decrease in higher-molecular-weight species. K19 is found mainly in simple epithelia (25, 41) but is also characteristic of simian virus 40-transformed human keratinocytes (17) and some cultured human squamous cell carcinomas (46). Thus, increased amounts of K19 in HPV-18-containing cells may be a significant change induced in primary human keratinocytes. The acidic 48-kDa (K16) and basic 56-kDa (K6) pair of cytokeratins are characteristic of hyperproliferative cells (43), and the acidic 50-kDa (K14) and basic 58-kDa (K5) pair are present in all keratinocytes (25, 41). All four of these cytokeratins are present in decreased amounts in the FE-A cell strain. Hronis et al. (17) reported the complete loss of these species from simian virus 40-transformed human keratinocytes. While there are some reports on changes in cytokeratin expression associated with cervical lesions either caused by HPV (38) or in general (2), it is not possible

to extrapolate changes observed in vivo in a different tissue to the in vitro model described here. However, on comparison with cultured keratinocytes under various conditions, the cytokeratin profile of FE-A cells most closely resembles that of a control culture stimulated to differentiate with TPA. This is also true of the involucrin amounts present in these HPV-18-containing keratinocytes. Thus, the differentiation markers of FE-A cells exhibit the characteristics of more differentiated keratinocytes but also those of a simple, transformed epithelium. FE-A cells exhibit these properties in their growth pattern in culture in that they are able to undergo some differentiation well before they reach confluence. However, when nearing confluence, a larger number of mitoses are present in FE-A cultures than in controls.

The above data confirm previous observations made after the introduction of HPV-16 into primary keratinocytes. In addition, we have been able to demonstrate the transformed nature of these cells by studying their calcium and TPA response. Normal mouse (16) and human epidermal (5) cells respond to an increase in calcium in the growth medium by stratification and terminal differentiation, particularly in the case of mouse keratinocytes. Similarly, TPA induces differentiation of normal human epithelial cells (27, 28, 45) and mouse epidermal cells (50) in a dose-dependent manner. However, it has been shown that initiated mouse epidermal cells in vitro (19) and in vivo (52), as well as transformed human epithelial cells (21, 26, 33), are able to resist or have a reduced response to differentiation signals such as calcium and TPA (for a review, see reference 26). FE-A cells are resistant to calcium as a stimulus for differentiation. Unlike control cultures, which become irreversibly committed to differentiation by treatment with calcium, FE-A cells are quiescent under these conditions and are able to resume proliferation upon removal of calcium from the growth medium. Thus, they appear to be blocked at an early stage in their maturation and are unable to terminally differentiate. This has been demonstrated by Yuspa et al. (53) for mouse keratinocytes infected with either Kirsten or Harvey sarcoma virus.

FE-A and FEH18L cells also showed marked resistance to TPA treatment. Some FE-A cells were able to terminally differentiate and be lost from the cultures; however, TPA-resistant cells were also evident in the cultures, and these continued to proliferate. It was evident that the TPA-resistant cells (FE-AP^r) were precursors of the sensitive cells, since subsequent treatment of an expanded population of FE-AP^r yielded a heterogeneous response in that differentiation-resistant and -sensitive cells were present within the cultures.

Tumorigenicity studies in nude mice have yielded no tumors to date with either FE-A, FE-AP^r, or FEH18L cells. These data are also in agreement with those of Durst et al. (8) and Pirisi et al. (32) for HPV-16-containing keratinocytes. This is perhaps not surprising, given that it is now generally accepted that carcinogenesis is a multistep process. This is certainly in keeping with epidemiological data on the frequency of HPVs in the general population and the occurrence of malignant neoplasia (see reference 7 for a review). Recent experiments have generated more keratinocyte cell strains transfected with HPV-18 or HPV-16 and should allow the consolidation of our initial observations with the FE-A and FEH18L cell strains. HPV-containing human keratinocyte cell strains will provide a suitable model system for the study of cofactors associated with HPV in carcinogenesis.

ACKNOWLEDGMENTS

We thank Harald zur Hausen for cloned HPV DNA and Philip Fleckman for helpful advice on initiating primary epithelial cultures. We are grateful to Denise Galloway for helpful discussion and comments and to Marci Wright for manuscript preparation.

This work was supported by a Public Health Service grant from the National Cancer Institute (CA-42792).

LITERATURE CITED

1. **Atkin, N. B.** 1986. Chromosome changes in preneoplastic and neoplastic genital lesions, p. 303–310. *In* R. Peto and H. zur Hausen (ed.), Viral etiology of cervical cancer (Banbury Report Vol. 21). Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Bobrow, L. G., C. A. Makin, S. Law, and W. F. Bodmer.** 1986. Expression of low molecular weight cytokeratin proteins in cervical neoplasia. J. Pathol. **148**:135–140.
3. **Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen.** 1984. A new type of papillomavirus DNA and its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. **3**:1151–1157.
4. **Boshart, M., and H. zur Hausen.** 1986. Human papillomaviruses in Buschke-Lowenstein tumors: physical state of the DNA and identification of a tandem duplication in the noncoding region of a human papillomavirus 6 subtype. J. Virol. **58**:963–966.
5. **Boyce, S. T., and R. G. Ham.** 1983. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J. Invest. Dermatol. **81**:33s–40s.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Broker, T. R., and M. Botchan.** 1986. Papillomaviruses: retrospectives and prospectives, p. 17–36. *In* M. Botchan, T. Grodzicker, and D. A. Sharp (ed.), Cancer cells, Vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Durst, M., R. T. Dzarlieva-Petrusevska, P. Boukamp, N. E. Fusenig, and L. Gissmann.** 1987. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene **1**:251–256.
9. **Durst, M., L. Gissmann, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80**:3812–3815.
10. **Durst, M. A. Kleinheinz, M. Hotz, and L. Gissmann.** 1985. The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumors. J. Gen. Virol. **66**:1515–1522.
11. **Fey, E. G., and S. Penman.** 1985. The morphological oncogenic signature: reorganization of epithelial cytoarchitecture and metabolic regulation by tumor promoters and by transformation, p. 81–100. *In* M. S. Steinberg (ed.), Developmental biology: A comprehensive synthesis, vol. 3. Plenum Press, New York.
12. **Gissmann, L., M. Boshart, M. Durst, H. Ikenberg, D. Wagner, and H. zur Hausen.** 1984. Presence of human papillomavirus in genital tumors. J. Invest. Dermatol. **83**:26s–28s.
13. **Gissmann, L., E.-M. De Villiers, and H. zur Hausen.** 1982. Analysis of human genital warts (condylomata acuminata) and other genital tumors for human papillomavirus type 6 DNA. Int. J. Cancer **29**:143–146.
14. **Gissmann, L. H., L. Wolnik, H. Ikenberg, U. Koldovsky, H. G. Schnurch, and H. zur Hausen.** 1983. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers. Proc. Natl. Acad. Sci. USA **80**:560–563.
15. **Harnden, D. G.** 1974. Viruses, chromosomes and tumors: the interaction between viruses and chromosomes, p. 152–190. *In* German (ed.), Chromosomes. John Wiley & Sons, New York.
16. **Hennings, H., D. Michael, C. Cheng, P. Steinert, K. Holbrook, and S. H. Yuspa.** 1980. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell **19**:245–254.
17. **Hronis, T. S., M. L. Steinberg, V. Defendi, and T.-T. Sun.** 1984.

Simple epithelial nature of some simian virus-40-transformed human epidermal keratinocytes. Cancer Res. **44**:5797–5804.

18. **Kaur, P., and J. K. McDougall.** 1987. Transformation of mouse cells by human papillomavirus type-6b and type-18 DNAs, p. 249–252. *In* B. M. Steinberg, J. L. Brandsma, and L. B. Taichman (ed.), Cancer cells, vol. 5. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Kulesz-Martin, M., B. Koehler, H. Hennings, and S. H. Yuspa.** 1980. Quantitative assay for carcinogen altered differentiation in mouse epidermal cells. Carcinogenesis **1**:995–1006.
20. **Lancaster, W. D., and C. Olson.** 1982. Animal papillomaviruses. Microbiol. Rev. **46**:191–207.
21. **Lechner, J. F., I. A. McClendon, M.-A. LaVeck, M. A. Shamsuddin, and C. C. Harris.** 1983. Differential control by platelet factors of squamous differentiation in normal and malignant human bronchial epithelial cells. Cancer Res. **43**:5915–5921.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Masui, T., J. F. Lechner, G. H. Yoakum, J. C. Willey, and C. C. Harris.** 1986. Growth and differentiation of normal and transformed human bronchial epithelial cells. J. Cell. Physiol. (Suppl.)**4**:73–81.
24. **McCance, D. J.** 1986. Human papillomaviruses and cancer. Biochim. Biophys. Acta **823**:195–205.
25. **Moll, R., W. W. Franke, and D. L. Schiller.** 1982. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell **31**:11–24.
26. **Parkinson, E. K.** 1985. Defective responses of transformed keratinocytes to terminal differentiation stimuli: their role in epidermal tumour promotion by phorbol esters and by deep skin wounding. Br. J. Cancer **52**:479–493.
27. **Parkinson, E. K., and A. Emmerson.** 1982. The effects of tumour promoters on the multiplication and morphology of cultured human epidermal keratinocytes. Carcinogenesis **3**:525–531.
28. **Parkinson, E. K., and A. Emmerson.** 1984. Non promoting hyperplasiogenic agents do not mimic the effects of phorbol 12-myristate 13-acetate on terminal differentiation of normal and transformed human keratinocytes. Carcinogenesis **5**:687–690.
29. **Parkinson, E. K., P. Grabham, and A. Emmerson.** 1983. A subpopulation of cultured human keratinocytes which is resistant to the induction of terminal differentiation-related changes by phorbol 12-myristate 13-acetate: evidence for an increase in the resistant population following transformation. Carcinogenesis **4**:857–861.
30. **Pfister, H.** 1984. Biology and biochemistry of papillomaviruses. Rev. Physiol. Biochem. Pharmacol. **99**:111–181.
31. **Pfister, H.** 1987. Human papillomaviruses and genital cancer. Adv. Cancer Res. **48**:113–147.
32. **Pirisi, L., S. Yasumoto, M. Feller, J. Doniger, and J. DiPaolo.** 1987. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J. Virol. **61**:1061–1066.
33. **Rheinwald, J. G., and M. A. Beckett.** 1980. Defective terminal differentiation in culture as a consistent and selectable marker of malignant human keratinocytes. Cell **22**:629–632.
34. **Rice, R. H., and H. Green.** 1979. Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. Cell **18**:681–694.
35. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.
36. **Shirasawa, H., Y. Tomita, K. Kubota, T. Kasai, S. Sekiya, H. Takamizawa, and B. Simizu.** 1986. Detection of human papillomavirus type 16 DNA and evidence for integration into the cell DNA in cervical dysplasia. J. Gen. Virol. **67**:2011–2015.
37. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
38. **Staquet, M. J., J. Viac, and J. Thivolet.** 1981. Keratin polypeptide modifications induced by human papillomaviruses (HPV). Arch. Dermatol. Res. **271**:83–90.
39. **Steinberg, M. L., and V. Defendi.** 1979. Altered patterns of growth and differentiation in human keratinocytes infected by simian virus 40. Proc. Natl. Acad. Sci. USA **76**:331–334.
40. **Steinberg, M. L., and V. Defendi.** 1983. Transformation and immortalization of human keratinocytes infected by simian virus 40. J. Invest. Dermatol. **81**:131s–136s.
41. **Tseng, S., M. J. Jarvinen, W. G. Nelson, J.-W. Huang, J. Woodcock-Mitchell, and T.-T. Sun.** 1982. Correlation of specific types of epithelial differentiation: monoclonal antibody studies. Cell **30**:361–372.
42. **Watts, S. L., W. C. Phelps, R. S. Ostrow, K. R. Zachow, and A. J. Faras.** 1984. Cellular transformation by human papillomavirus DNA in vitro. Science **225**:634–636.
43. **Weiss, R. A., R. Eichner, and T.-T. Sun.** 1984. Monoclonal antibody analysis of keratin expression in epidermal diseases: 48- and 56-KD keratins as molecular markers for hyper-proliferative keratinocytes. J. Cell Biol. **98**:1397–1406.
44. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Orlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.
45. **Willey, J. D., C. E. Moser, Jr., J. F. Lechner, and C. C. Harris.** 1984. Differential effects of 12-*O*-tetradecanoyl phorbol-13-acetate on cultured normal and neoplastic human bronchial epithelial cells. Cancer Res. **44**:5124–5126.
46. **Wu, Y.-J., and J. G. Rheinwald.** 1981. A new small (40kD) keratin filament protein made by some cultured human squamous cell carcinomas. Cell **25**:627–637.
47. **Yasumoto, S., A. L. Burkhardt, J. Doniger, and J. A. DiPaolo.** 1986. Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J. Virol. **57**:572–577.
48. **Yee, C., I. Krishnan-Hewlett, C. C. Baker, R. Schlegel, and P. M. Howley.** 1985. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am. J. Pathol. **119**:361–366.
49. **Yoakum, G. H., J. F. Lechner, E. W. Gabrielson, B. E. Korba, L. Malan-Shibley, J. C. Willey, M. G. Valerio, A. M. Shamsuddin, B. F. Trump, and C. C. Harris.** 1985. Transformation of human bronchial epithelial cells transfected by Harvey *ras* oncogene. Science **227**:1174–1179.
50. **Yuspa, S. H., T. Ben, H. Hennings, and U. Lichti.** 1980. Phorbol ester tumor promoters induce epidermal transglutaminase activity. Biochem. Biophys. Res. Commun. **97**:700–708.
51. **Yuspa, S. H., T. Ben, H. Hennings, and U. Lichti.** 1982. Divergent responses in epidermal basal cells exposed to the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate. Cancer Res. **42**:2344–2349.
52. **Yuspa, S. H., and D. L. Morgan.** 1981. Mouse skin cells resistant to terminal differentiation associated with initiation of carcinogenesis. Nature (London) **293**:72–74.
53. **Yuspa, S. H., W. Vass, and E. Scolnick.** 1983. Altered growth and differentiation of cultured mouse epidermal cells infected with oncogenic retrovirus: contrasting effects of viruses and chemicals. Cancer Res. **43**:6021–6030.
54. **Zur Hausen, H.** 1977. Human papillomaviruses and their possible role in squamous cell carcinomas. Curr. Top. Microbiol. Immunol. **78**:1–30.